

# Stability of Outcrossing Rates in *Eucalyptus globulus* Seedlots

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## Summary

*Eucalyptus globulus* has a mixed mating system and selfing leads to severe inbreeding depression. Thus, outcrossing rate is an important measure of seed quality in this species. Outcrossing rates were estimated on the frequency of a rare recessive chlorophyll deficient mutant in open pollinated seed. Variation of outcrossing rates across different flowering seasons, seed sizes and storage times was assessed in *E. globulus* seedlots. Although there was a significant difference between the trees studied, outcrossing rates within a tree were found to be stable across four flowering seasons and three seed sizes. Storage either on the tree or in the laboratory did not alter the outcrossing rate in a consistent manner. The stability of outcrossing rates with season of pollination, age and size of seed is noteworthy as these factors may vary widely in multi-tree seedlots used for plantation establishment.

**Key words:** Selfing, seed size, seed age.

## Introduction

*Eucalyptus globulus* Labill. (sensu BROOKER, 2000) is the premier *Eucalyptus* species for pulpwood plantations in many temperate countries around the world (ELDRIDGE *et al.*, 1993). As it is often difficult to propagate the species vegetatively (TIBBITS *et al.*, 1997), open pollinated seed orchards have been established for the production of improved seed. However, as these orchards rely on open-pollination, they can fail to produce high quality seed due to the mixed mating system of *E. globulus*. Although *E. globulus* is preferentially outcrossing, selfing is not uncommon and the outcrossing rates in *E. globulus* seed orchards have been reported to range from 77% (MONCUR *et al.*, 1995) to 92% (RUSSELL *et al.*, 2001). Selfed progenies suffer significant inbreeding depression in growth and volume when compared to outcrossed progenies (HARDNER and POTTS, 1995). Because inbreeding depression can deleteriously affect productivity of plantations it is important to investigate the factors that may affect the outcrossing rate of *E. globulus*. For example, it has been observed in other serotinous species, including *Eucalyptus delegatensis* (MORAN and BROWN, 1980), *Pinus contorta* (HAMRICK, 1989) and *Pinus banksiana* (CHELIAK *et al.*, 1985; SNYDER *et al.*, 1985), that outcrossing rates have increased in seed that was retained longer on the tree. Such observations could be due to chance seasonal variation or the germination of selfed (or inbred) seed decreasing over time through seed aging.

Another major concern for plantation growers is the practise of segregating seed into size classes to improve uniformity in germination and seedling growth. Larger eucalypt seeds germinate faster, produce larger seedlings and show higher seedling survival rate (BALLONI *et al.*, 1978; BATTAGLIA, 1993; BODEN, 1961; LOPEZ *et al.*, 2003; MARTINS-CORDER *et al.*, 1998; WATSON *et al.*, 2001). However this has raised concerns that slower growth from smaller seed may reflect high levels of self-ferti-

sation which may affect growth later. Selfed seed has been found to be smaller in several other species with mixed mating system, including *Dombeya acutangula* ssp. *acutangula* (GIGORD *et al.*, 1998), *Banksia spinulosa* (VAUGHTON and RAMSEY, 1997), *Phormium tenax* (CRAIG, 1989) and *Hibiscus moscheutos* (SNOW and SPIRA, 1993). The outcrossing rates of different seed size classes have not been examined previously in *E. globulus*.

The present study investigates the direct effect of season, size and seed age on outcrossing rates in *E. globulus* by comparing the frequency of albino progeny in seed samples from three separate harvests, with seed aged both on the tree and in the laboratory. While previous studies usually used allozyme markers to estimate outcrossing rates (CHELIAK *et al.*, 1985; EL-KASSABY *et al.*, 1993; MORAN and BROWN, 1980; SNYDER *et al.*, 1985), this study used a single locus morphological marker. The marker was identified by PATTERSON *et al.* (2000) in two *E. globulus* trees that carry mutations in separate genes, which result in chlorophyll deficient seedlings when the mutation is homozygous. This marker is expressed at the cotyledonary stage, which enables a quick and easy estimate of the outcrossing rate from the deviation of the proportion of the albino germinants from a one to three ratio. This method allows large sample sizes to be screened for outcrossing rate and has been shown to be consistent with multi-locus isozymes estimates not only in *E. globulus* (PATTERSON *et al.*, 2000) but also in *Pinus ponderosa* (MITTON *et al.*, 1981).

## Materials and Methods

### Seed collection and germination

Open pollinated seed was collected from two trees located in remnant native stands on farmland in southern Tasmania (tree numbers 309 and 513), that were used in the study of chlorophyll mutant inheritance by PATTERSON *et al.* (2000). Seed was collected in 2000, 2001 and 2002. One and two year old capsules were randomly harvested from around the lower canopy of each tree. Thus seed was available from 1998, 1999, 2000 and 2001 flowering seasons since seed takes one year to mature in this *Eucalyptus* species. Capsules from each tree, harvest and flowering year combination were air dried as separate lots and then the seed removed from the capsules and sieved into three size fractions greater than 1.7 mm, 1.7 mm to 1.4 mm, and 1.4 mm to 1 mm. Within a month of harvest, the seedlots were sub-sampled and seed germinated in order to assess the frequency of chlorophyll mutants. In years 2000 and 2001 excess seed was stored and used in germination experiments of subsequent years to examine the effects of storage in ambient conditions.

For germination, samples of each seedlot were arranged in a randomised block design with each seedlot being represented by 2 to 9 replicates depending on seed availability. The seed sown from the last two years harvests was counted to allow for germination rate estimates. The seed sample from a given seedlot was evenly spread on the surface of potting mix in a seed punnet, and the punnet constituted the plot in the experi-

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mental design. The punnets were randomly arranged on 28 °C heat beds in a glasshouse. Watering was provided by overhead misting, with the air temperature unregulated. Punnets were lightly sprayed with 1g L<sup>-1</sup> of a benlate fungicide solution. Punnets were monitored regularly and scored for normal (green) or albino (yellow-pink) germinants with fully expanded cotyledons for up to 40 days following sowing. The seed from the 2002 harvest was spread evenly across the surface of vermiculite in sealable punnets and randomly arranged in a controlled growth cabinet at 25 °C with 18 hours of light. Otherwise treatments were similar to previous years.

#### Data analysis

Frequencies of normal (green) seedlings and albino seedlings were calculated over all the experiments and replicates using the PROC FREQ procedure in SAS (version 8). Contingency  $\chi^2$  tests were used to determine differences between the frequencies of green and albino seedlings between trees, seasons, size classes and storage times. Selfing rate was estimated using the proportion of albino seedlings in a punnet which was multiplied by four, based on the assumption that albinos are only produced by selfing (PATTERSON *et al.*, 2000). Outcrossing was then calculated by subtracting the selfing rate from one and expressing it as a percentage. Significant variation in the frequency of the albino seedlings was assumed to represent significant variation in outcrossing rates.

#### Results and Discussion

The two trees differed significantly in their outcrossing rate ( $\chi^2_1 = 10.36$ ;  $P < 0.0013$ ). Tree 309 had a outcrossing rate of 48% and tree 513 had a outcrossing rate of 38% (averaged across all treatments). The low outcrossing rates are consistent with the collection of seed in the lower canopy which is known to have a lower outcrossing rate than seed collected in the upper canopy in self-compatible trees (PATTERSON *et al.*, 2001). These outcrossing rates would be an indication of poor quality seed in forestry, but for our purpose they were ideal since they gave us scope for studying the factors that cause variation in selfing rate. The outcrossing rates were stable across flowering seasons for both trees (Table 1), with no significant differences found in the frequency of albinos between four seedlots for tree 309 ( $\chi^2_3 = 3.12$ ;  $P < 0.3736$ ) and six seedlots for tree 513 ( $\chi^2_5 = 7.57$ ;  $P < 0.1813$ ). The outcrossing rates across a number of flowering seasons and capsule ages have not been examined in *Eucalyptus globulus* previously, however it has been studied in two other eucalypt species, *E. delegatensis* (MORAN and BROWN,

1980) and *E. stellulata* (BROWN *et al.*, 1985). MORAN and BROWN (1980) found that the oldest of three seed crops of *E. delegatensis* had the highest outcrossing rate. In the study of *E. stellulata* eight seed crops were collected over three years. Temporal variation was found. One particular flowering season had a high selfing rate, and this decreased when the seed remained on the tree (BROWN *et al.*, 1985).

Similar outcrossing rates have been determined for these same two trees (309 and 513) in previous studies. PATTERSON *et al.* (2000) used the proportion of albino progeny from the 1997 flowering season to calculate an outcrossing rate of 49.2% for tree 309 and 41% for tree 513. Using isozymes, outcrossing was estimated to be  $52 \pm 11\%$  for tree 309. The outcrossing rate of tree 309 was unchanged (49%) for the 1998 season. This seasonal stability is unexpected as outcrossing rate in *E. globulus* was believed to be affected by both pollinator (MONCUR *et al.*, 1995) and flower abundance, two factors regarded as variable (HINGSTON, 2002). Although this study did not specifically monitor pollinator activity or flowering, the flowering years encompassed by this study included some of the lowest as well as the highest flowering seasons recorded in *E. globulus* trees in south-eastern Tasmania over a 18 year period (PAUL THLYARD, pers. com). Thus our results suggest that pollinator activity and flower abundance may have little effect on outcrossing rate of these two trees, rather the self-incompatibility level of a tree is probably the primary determinant of its outcrossing rate.

Storage under laboratory conditions had varied effects on outcrossing rates. The outcrossing rates of seed from tree 513 did not differ significantly ( $\chi^2_2 = 0.22$ ;  $P < 0.8962$ ), whether it had been left in the laboratory for one or two years (Table 2). However, the outcrossing rates increased significantly for tree 309 when the seed had been stored at room temperature in the laboratory for two years ( $\chi^2_2 = 11.49$ ;  $P < 0.0032$ ). This was associated with a reduction in the germination from 87.9% after one year of storage to 80.7% after two years of storage. While the overall tests between seedlots collected on each tree (Table 1) showed no significant differences, the specific comparison of seedlots of tree 513 from the 1999 and 2000 flowering seasons which had been aged for an additional year on the tree did show a significant increase in outcrossing rate for the 2000 flowering season ( $\chi^2_1 = 4.01$ ;  $P < 0.0452$ ) but not the 1999 season ( $\chi^2_1 = 0.03$ ;  $P < 0.8523$ ). Seeds deteriorate during storage and eventually die due to aging (WU *et al.*, 1998) and it has been well documented that chromosomal aberrations and point mutations occur during seed storage (ROOS, 1982). DNA repair has been suggested to occur in seeds stored in moist conditions only (ROBERTS, 1988), as repair is unlikely to occur in dry storage as normal metabolism is not possible. In each case where we obtained a significant difference in outcrossing rate with seed aging, our sample size was low, but this involved increased outcrossing rate. Aging effects on outcrossing rate

Table 1. – Total number of seedlings, percentage of albinos and outcrossing rates for *E. globulus* seedlots collected from different age capsules from two different trees over three harvests.

Tree	Harvest year	Flowering season	Seed age	Total		Outcrossing
				number of seedlings	% Albino	
309	2000	1999	1	2844	13.3	46.8
		1998	2	532	11.1	55.6
	2002	2001	1	603	12.4	50.2
		2000	2	633	14.4	42.5
513	2000	1999	1	1642	15.2	39.4
		1998	2	1818	15.3	38.8
	2001	2000	1	373	18.2	27.1
		1999	2	1063	15.4	38.3
	2002	2001	1	645	18.1	27.4
		2000	2	601	13.5	46.1

Table 2. – Total number of seedlings, percentage of albinos and outcrossing rate for *E. globulus* seedlots collected from two different trees and stored in laboratory conditions for one and two years.

Tree	Storage time (years)	Total number of seedlings	% Albino	Outcrossing
309	0	2844	13.3	46.8
	1	918	12.3	50.8
	2	242	5.8	76.8
513	0	1642	15.2	39.4
	1	1103	14.7	41.2
	2	209	15.8	36.8

Table 3. – Total number of seedlings, percentage of albinos and outcrossing rate for *E. globulus* seedlots collected from two different trees in three seed size classes.

Tree	Size Class (mm)	Total number of seedlings	% Albino	Outcrossing %
309	>1.7	1563	13.2	47.0
	>1.4<1.7	2589	12.8	48.7
	>1<1.4	460	13.9	44.4
513	>1.7	2301	15.7	37.2
	>1.4<1.7	3382	15.8	37.0
	>1<1.4	459	13.7	45.1

require further study. Nevertheless from a practical perspective, any commercial benefit gained from improved outcrossing rate in aged seed would be countered by the negative effects of reduced germination on nursery profitability.

Outcrossing rate did not differ significantly between the three different size classes in either tree (Table 3; tree 309  $\chi^2_2 = 0.47$ ;  $P < 0.7915$  and tree 513  $\chi^2_2 = 1.3$ ;  $P < 0.521$ ). Nurseries normally segregate *E. globulus* seed into different seed sizes to promote even germination and seedling growth (WATSON *et al.*, 2001). As previously mentioned this practise based on research that has found that larger seeds germinate faster and better, produce larger seedlings and show a higher seedling survival rate. However there are concerns that slower growth from smaller seed may reflect high levels of self-fertilisation which may affect growth later. A previous study examined the genetic diversity of three size classes of seeds in *Eucalyptus globulus* using isozymes and found that the smallest seeds had the lowest fixation index which would indicate less inbreeding (MARTINS-CORDER *et al.*, 1998). It was suggested that this could be due to higher mortality of selfed progenies in the small seed class. However, this is inconsistent with our results and those of HARDNER and POTTS (1995). While artificially selfed seed did have a higher percentage of inviable seed (seed with flattened testa) than outcrossed seed in the latter study, the germination percentage and germination rate of the seed which was classified as viable did not differ with cross type. Thus our results are different to that of other species where it is has been observed that selfed seed can be smaller (CRAIG, 1989; VAUGHTON and RAMSEY, 1997).

In summary, this study suggests that there is no clear advantage or disadvantage in allowing seed to age on the tree, or under laboratory conditions for a few years. Outcrossing rate appears to be stable across seasons, suggesting seed producers can collect seed whenever it is required and it can then be segregated into separate seed size classes to ensure more even germination. The smallest seed class can still be used by nurseries, after discounting for low germination rate, as its outcrossing rate is likely to be the same as that of the larger seeds.

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